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   Method and construct for inhibition of cell migration

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Title: Method and Construct for inhibition of cell migration

FIELD OF THE INVENTION

The invention is in the field of therapeutic means and therapeutic methods for treatment of diseases in which cell migration and/or tissue remodeling occurs. Furthermore, 5 the invention is in the field of biotechnology, in particular recombinant DNA technology and gene therapy.

BACKGROUND OF THE INVENTION

Migration of cells is an essential step in many physiological and pathological processes in which tissue 10 remodeling occurs, such as tumor metastasis, wound healing, restenosis, angiogenesis or rheumatic arthritis. Migrating cells have to pass through the surrounding extracellular matrix. Limited proteolytic degradation of the components of the extracellular matrix is often seen during cell migration. 15 To mediate this cell migration migrating cells produce, or recruit from their direct environment, proteolytic enzymes, such as plasminogen activators, metalloproteinases or elastases. Induction of cell migration e.g. during tumor metastasis or wound healing often correlates with the 20 induction of the production of these enzymes.

Although the involvement of proteolytic enzymes in cell migration under pathophysiological conditions is well accepted, little attempts have been made to inhibit cell 25 migration by inhibiting these proteolytic enzymes. A possible explanation for the limited use of protease inhibitors is the fact that these proteolytic enzymes are involved in many processes both pathological and physiological (including fibrinolysis, wound healing, growth factor activation etc.) 30 and that inhibition of these protease systems by systemically applied protease inhibitors might have either strong side effects or may lead to a diffusion or clearance of the inhibitory compounds without having a strong effect on the local cell migration processes.

Another problem in the use of protease inhibitors to interfere in cell migration and tissue remodeling is that proteases mediating these processes can bind to receptors at the cell surface. In this way the proteolytic enzymes might

5 be active locally in a pericellular microenvironment where they are protected against the action of the present inhibitors.

It has been disclosed that conjugates between the receptor binding part of u-PA (the aminoterminal fragment or

10 ATF) and urinary trypsin inhibitor produced in vitro, inhibit migration of tumor cells in vitro (Kobayashi, Gotoh, Hirashima, Fujie, Sugino and Terao, Inhibitory effect of a conjugate between human urokinase and urinary trypsin inhibitor on tumor cell invasion in vitro. J. Biol. Chem.

15 (1995) 270, 8361-8366). The conjugate these authors have used is made synthetically by mixing the isolated ATF fragments with the trypsin inhibitor.

A comparable construct consisting of a receptor binding u-PA fragment and its inhibitor PAI-2, to be produced

20 recombinantly in yeast, has been described to inhibit tumor cell migration in WO 92/02553 (PCT/GB91/01322). In this way they have made a protease inhibitor that can bind to a specific receptor at the cell surface, the urokinase receptor, and this inhibitor can inhibit cell migration (in

25 vitro). As to the use of these constructs in vivo, a problem is the application to and the prolonged presence at the site of desired action in vivo.

SUMMARY OF THE INVENTION

30 This invention provides a recombinant nucleic acid molecule comprising a vector useful for transfection or transduction of mammalian, e.g. human, cells, wherein said vector contains a nucleic acid insertion encoding an expressible hybrid polypeptide or protein which comprises a

35 domain with a binding function and a domain with an effector function. Herein, the domain with a binding function

preferably comprises a receptor binding domain, and the domain with an effector function preferably has enzymatic activity, most preferably protease inhibitor activity.

Preferably, the receptor binding domain is selected from the group consisting of urokinase receptor binding domain of urokinase, receptor binding domain of epidermal growth factor, receptor associated protein that binds to LDL Receptor related protein (α_2-macroglobulin receptor) and VLDL Receptor.

Preferably, the domain with an effector function has protease inhibitor activity and comprises a protease inhibitor or active part thereof, said protease inhibitor being selected from the group consisting of (bovine) pancreatic trypsin inhibitor, (bovine) splenic trypsin inhibitor, urinary trypsin inhibitor, tissue inhibitor of matrix metalloproteinase 1, tissue inhibitor of matrix metalloproteinase 2, tissue inhibitor of matrix metallo-proteinase 3, and elastase inhibitor. The domain with an effector function may comprise (an active part of) two or more different protease inhibitors, or two or more copies of (an active part of) a protease inhibitor, or both.

Preferably, the vector is selected from the group consisting of viral and non-viral vectors useful for trans-fection or transduction of mammalian cells. The vector may be an adenovirus vector or a retrovirus vector useful for trans-fection or transduction of human cells.

The nucleic acid insertion encoding an expressible hybrid polypeptide or protein may be under the control of a cell- or tissue-specific promoter, such as an endothelial cell-specific promoter, or a vascular smooth muscle cell-specific promoter, or a liver-specific promoter.

This invention furthermore provides a process for preventing local proteolytic activity, extracellular matrix degradation, cell migration, cell invasion, or tissue remodeling, comprising transfecting or transducing the cells involved or cells in their environment with a recombinant

nucleic acid molecule as defined herein to obtain local
expression of the hybrid polypeptide or protein encoded by
said nucleic acid molecule.

Also, this invention provides a process for
5   producing a hybrid polypeptide or protein which comprises a
domain with a binding function and a domain with an effector
function, comprising transfecting or transducing mammalian
cells with a recombinant nucleic acid molecule as defined
herein to obtain expression of the hybrid polypeptide or
10 protein encoded by said nucleic acid molecule, and optionally
recovering the hybrid polypeptide or protein produced.

BRIEF DESCRIPTION OF THE DRAWINGS
        Figure 1 schematically depicts the plasmids pCRII-
15 uPA (left) and pCRII-ATF (right).
        Figure 2 schematically depicts the plasmid pCRII-
ATF-BPTI.
        Figure 3 schematically depicts the plasmid pMAD5-
ATF-BPTI.
20   Figure 4 shows the results of proteolytic matrix
degradation experiments.

DETAILED DESCRIPTION OF THE INVENTION
        The present invention relates to the use of hybrid
25 proteins in which a receptor binding domain is linked to a
functional protein in order to induce a local action of this
protein and to prevent systemic effects and/or diffusion. In
particular this invention relates to such hybrid proteins
that might be produced by a subset of cells as target cells
30 after transfection or transduction with expression vectors.
More specifically the invention relates to the use of such
expression vectors, coding for hybrid proteins consisting of
a receptor binding domain and a protease inhibitor domain,
for the prevention of cell migration and tissue remodeling by
35 inhibition of proteases at the surface of migrating or
invading cells.

The method and construct described in the present invention can be applied as therapy in diseases in which cell migration and/or tissue remodeling occurs.

The present invention addresses the solution of several negative aspects involved in the above described use of inhibitors according to the prior art:

- High local concentrations of hybrid proteins in the direct environment of the target cells can be obtained by production of the protein by the migrating cells themselves or cells in their immediate environment. This production can be induced by transfection or transduction of a certain subset of the cell population with a suitable vector encoding the hybrid protein. For this purpose, one may use recombinant adenoviral vectors, retroviral vectors, plasmid vectors, etc.

- Diffusion of the inhibitor and systemic side effects are prevented by binding the hybrid protein (by its receptor binding domain) to the cell surface of the target cell. Local expression of this hybrid protein also contributes to the reduction of systemic side effects, while the negative effect of diffusion of the protein is reduced by the production at the site where action is required. The local expression of the hybrid protein in specific subpopulations of cells, e.g. endothelial cells prone to migrate during angiogenesis, can be enhanced using cell type-specific or tissue-specific expression vectors, in which the expression of the protein is under control of a promoter with cell type-specific or tissue-specific regulatory elements.

- Binding of a protease inhibitor to a cell surface receptor can locate the inhibitor close to its molecular target, the cell surface bound proteolytic enzyme. Local inhibition of the proteolytic activity in the pericellular microenvironment may be achieved in this way.

- Binding of a protease inhibitor to a cell surface receptor for a proteolytic enzyme, such as the urokinase receptor, may have an additional inhibitory effect. It prevents the binding of the proteolytic enzyme to its

receptor, and thus strongly reduces the action of this enzyme as has been shown for blocking the binding of u-PA to its receptor which can strongly inhibit cell migration.

Hybrid proteins, for which the expression vectors (e.g. adenoviral or retroviral expression vectors) contain the encoding DNA sequences, might contain a region that binds to a cell surface receptor and that is not subsequently internalized. Receptor binding domains that can be used for this purpose are e.g. the u-PAR binding domain of urokinase plasminogen activator, the receptor binding domain of epidermal growth factor, the receptor associated protein (RAP) that binds to the LDL-R related protein (LRP), also called α_2-macroglobulin receptor, and the VLDL-receptor.

The inhibitor part of the encoded hybrid protein might consist of various protease inhibitors such as bovine pancreatic trypsin inhibitor, also called aprotinin or Trasylol©, other trypsin inhibitors such as urinary trypsin inhibitor, inhibitors for matrix-degrading metalloproteinases such the tissue inhibitors of metalloproteinases TIMP-1, TIMP-2 and TIMP-3, or variants thereof. Also inhibitors for other proteases like elastase are very suitable for being incorporated into the expression vector containing the DNA sequences encoding the hybrid proteins. Multiple copies of the DNA sequences encoding the functional protein part of the hybrid protein e.g. the inhibitor part, or combinations of different inhibitors or derivatives thereof might be incorporated into the DNA construct in the expression vector.

Another very attractive possibility would be to use such an expression vector encoding hybrid receptor binding protein to apply any functional protein that should exert its action in the local environment of the target cell, e.g. a protease involved in the activation of a growth factor or an other e.g. vasoregulatory component.

The action of the functional protein or protein domains of the hybrid protein is localized to the direct microenvironment of the target cells by binding of the

receptor binding domain to a receptor at the surface of the
target cells. Production of the hybrid protein in the direct
environment of the target cells or even by the target cells
themselves can be obtained by transfection or transduction of
5   these cells by the use of expression vectors that might be
based on a non-viral or an adeno- or retroviral vector
system. Expression in specific cell or tissue types might be
achieved by the use of specific promoter elements in the
expression vectors. For example, for endothelial cell-
10   specific expression (elements of) the promoter region of the
human or murine pre-pro-endothelin gene (HUMEDN1B and
MMU07982, respectively, GENBANK) can be used, for vascular
smooth muscle cell-specific expression (elements of) the
promoter region of the human vascular smooth muscle α-actin
15   gene (HUMACTSA, GENBANK) can be used, and for liver-specific
expression the promoter of the human albumin gene (HUMALBGC,
GENBANK) can be used.

Local delivery of these vectors might be obtained
using various commonly used methods, including catheters,
20   topically applied gels containing the vectors or targeted
delivery systems. For site-specific delivery to the vessel
wall, e.g. to prevent restenosis and vessel wall remodeling
after angioplasty, special catheters can be used. At the
moment double balloon catheters, channeled balloon catheters,
25   multiple needle catheters and balloon catheters coated with a
vector containing a hydrogel are being used for vessel wall-
specific delivery. Other ways to deliver the vectors directly
into the vessel wall are the use of stents coated with vector
containing coatings, topical application of vector containing
30   hydrogels to the outside of the blood vessel or ex vivo
delivery directly into the blood vessel during trans-
plantation surgery. Ex vivo transduction of proliferating
cells using retroviral vectors followed by a reinjection may
also be used to deliver the vector constructs at the site
35   where their action is required.

The present application will be described herein-
after in further detail, while referring to the following
examples. It is to be noted that these examples merely serve
to illustrate the invention, not to restrict it.

5

EXAMPLE 1

An expression plasmid encoding the aminoterminal
fragment of urokinase plasminogen activator (u-PA), amino
acids 1-138, hereafter referred to as ATF, can be constructed
10 by deleting the DNA sequences encoding amino acids 139 till
401 in an expression plasmid for the full length u-PA using a
polymerase chain reaction (PCR) with the following oligo-
nucleotides: 5'-cccgggcttttttccatctgcgcagtc-3' and
5'-agggtcaccaaggaagagaatggc-3'. After amplification by PCR
15 the newly formed DNA fragment can be circularized by ligation
to restore the circular character of the expression plasmid.
In this way an expression plasmid encoding the ATF and the C
terminal last 11 amino acid residues including the stop codon
can be constructed.

20 The sequence of the thus formed DNA construct enco-
ding the u-PA ATF fragment then is determined and compared
with the predicted sequence as a control for possible
mutations introduced during the construction procedure.

The construction pCRII-ATF from pCRII-uPA using PCR
25 is shown in Figure 1. In figure 1, the area indicated between
the lines was removed during the PCR amplification, resulting
in the ATF plasmid. The plasmid pCRII-uPA is shown to the
left, plasmid pCRII-ATF to the right.

30 EXAMPLE 2

DNA fragments encoding amino acid residues 36-93 of
bovine pancreatic trypsin inhibitor (BPTI) and the homologous
amino acid residues of bovine spleen trypsin inhibitor (BSTI)
can be isolated by performing a PCR reaction on genomic DNA
35 isolated for bovine aortic endothelial cells using the
following oligonucleotides: 5'-_tcgcga_cctgacttctgcctagagc-3'

covering nucleotides 2509 to 2533 (with modifications,
indicated in *italics*, in the 5' region of the oligonucleotide
to introduce a NruI site (underlined) for cloning purposes)
of the BPTI gene according to the published sequence
5   (GENBANK, BTBPTIG), and nucleotide 2442 to 2462 of the BSTI
gene according to the published sequence (GENBANK, BTBSTIG)
and 5'-*ggtcaccc*aggg*ccca*atattaccacc-3' covering nucleotides
2677 to 2704 of the BPTI gene and 2610 to 2636 of the BSTI
gene (modified in the indicated nucleotides (*italics*) to
10 introduce a BstEII and a SspI site respectively
(underlined)). The amplified DNA fragments then were cloned
into an appropriate plasmid vector, pCRII or pUC13, and then
the exact sequence of the amplified DNA fragments in the
isolated clones was analyzed to differentiate between BPTI
15 and BSTI which have a very high degree of homology.

EXAMPLE 3

         The DNA fragment encoding amino acids 1 to 207 of
the human tissue inhibitor of metalloproteinase type 1 is
20 isolated by performing a reverse transcriptase polymerase
chain reaction on total RNA isolated from human foreskin
fibroblasts by using the following oligonucleotides
5'-agagagacaccagagaacccaccat-3' covering nucleotides 41 to 65
of the human TIMP-1 cDNA (according to the sequence in
25 GENBANK HSTIMPR) and 5'-tcattgtccggaagaaagatgggag-3' covering
nucleotides 740 till 765. The amplified DNA fragment was
cloned into an appropriate host vector, pUC13, and then the
exact sequence of the amplified DNA fragment in the isolated
clones was analyzed.
30

EXAMPLE 4

         For construction of a recombinant adenovirus
containing sequences encoding the ATF.BPTI hybrid protein,
this sequence is cloned in the adenoviral vector construction
35 adapter and expression plasmid pMAD5. This plasmid contains
part of the wildtype adenovirus type 5 DNA sequences, a Major

Late Promoter (MLP), and a poly-adenylation (polyA) signal
and can be used as either an expression vector or a shuttle
vector to construct a recombinant adenovirus. This plasmid
was derived from plasmid pMLP10 as follows. First pMLP10-lin

5   was constructed by insertion of a synthetic DNA fragment with
unique sites for the restriction endonucleases MluI, SplI,
SnaB1, SpeI, AsuII and MunI into the HindIII site of pMLP10.
Subsequently, the adenovirus BglII fragment spanning nt 3328
to 8914 of the Ad5 genome was inserted into the MunI site of

10   pMLP10-lin. Finally, the SalI-BamHI fragment was deleted to
inactivate the tetracycline resistance gene, resulting in
plasmid pMAD5. To clone the ATF.BPTI sequence into the pMAD5
plasmid between the MLP promoter and the polyA signal the
following strategy has been followed.

15   Starting from a pCRII plasmid in which a 1373 base
pair fragment of the uPA cDNA was cloned, a PCR reaction with
the oligonucleotides 5'-_ccc_ggg_ctttttt_ccatctgcgcagtc-3' (SmaI
site underlined and nucleotides changed in *italics*) and
5'-ag_ggtcacc_aaggaagagaatggc-3' (BstEII site underlined and

20   nucleotides changed in *italics*) was performed as described in
example 1 to make a pCRII-ATF plasmid (see figure 1).
Subsequently this pCRII-ATF plasmid was digested with the
restriction enzymes SmaI and BstEII. In parallel the pCRII-
BPTI plasmid was digested with the restriction enzymes NruI

25   and BstEII and the BPTI containing fragment was cloned into
the pCRII-ATF plasmid (see figure 2). The construction pCRII-
ATF-BPTI is shown in Fig. 2.

          In a next step the ATF-BPTI sequence was cloned
into pMAD5. This was done by digestion of the pCRII-ATF-BPTI

30   plasmid with the restriction enzymes EcoRV and SpeI,
isolation of the ATF-BPTI encoding DNA fragment and cloning
of this fragment into the SnaBI and SpeI digested pMAD5
plasmid. The cloning was tested by restriction analysis and
sequence analysis.

35   The pMAD5-ATF-BPTI shuttle vector for the construc-
tion of ATF-BPTI adenoviral vector is shown in Figure 3.

EXAMPLE 5

In a similar way as described in example 4 for pMAD5-ATF-BPTI a plasmid containing the BSTI-gene (pMAD5-ATF-BSTI) was constructed using the pCRII-BSTI plasmid instead of
5 the pCRII-BPTI plasmid.

EXAMPLE 6

For construction of a recombinant adenovirus containing sequences encoding the ATF-TIMP1 hybrid protein,
10 this sequence is cloned in the pMAD5 expression plasmid. This plasmid contains part of the wildtype adenovirus type 5 DNA sequences, a Major Late Promoter (MLP), and a polyadenylation (polyA) signal and can be used as either an expression vector or a shuttle vector to construct a recombinant adenovirus. To
15 clone the ATF-TIMP1 sequence into the pMAD5 plasmid between the MLP promoter and the polyA signal, the following strategy has been followed.

Starting from a pCRII plasmid in which a 1373 base pair fragment of the uPA cDNA was cloned, a PCR reaction with
20 the oligonucleotides 5'-cccgggcttttttccatctgcgcagtc-3' and 5'-aggtcaccaaggaagagaatggc-3' was performed as described in example 1 to make a pCRII-ATF plasmid (see figure 1). Subsequently this pCRII-ATF plasmid was digested with the restriction enzymes SmaI and BsteII.

25 In parallel a fragment of the cDNA of TIMP1 in pUC13-TIMP1 encoding amino acid residues 1 to 184 of the mature protein, but lacking the signal peptide and the stop codon, was amplified using the following oligonucleotides 5'-tcgcgatgcacctgtgtccacc-3' and
30 5'-ggtcacccaaatattggctatgtgggaccgcaggg-3'. These oligo-nucleotides contain recognition sites for the restriction enzymes NruI (first oligonucleotide, underlined) and BstEII and SspI respectively (second oligonucleotide, underlined); these sites are needed for the cloning procedure.

35 The amplified DNA fragment was cloned into a pCRII vector and called pCRII-TIMP1. This vector was subsequently

12

digested with the restriction enzymes NruI and BsteII and the
TIMP1 containing DNA fragment was cloned into the pCRII-ATF
plasmid (see figure 1).

5   In a next step the ATF-TIMP sequence was cloned
into pMAD5. This was done by digestion of the pCRII-ATF-TIMP
plasmid with the restriction enzymes EcoRV and SpeI,
isolation of the ATF-TIMP encoding DNA fragment and cloning
of this fragment into the SnabI and SpeI digested pMAD5
plasmid. The cloning was tested by restriction analysis and
10   sequence analysis.

      EXAMPLE 7
            Vectors encoding hybrid proteins containing
multiple copies of the BPTI unit coupled to the ATF domain
15   have been constructed. To construct these multiple BPTI
vectors, the following strategy is followed.
            The pMAD5-ATF-BPTI described in EXAMPLE 4 is
digested with the restriction enzymes SspI and BstEII. In
this way the vector is opened exactly in the open reading
20   frame at the end of the BPTI sequence. The pCRII-BPTI plasmid
described in EXAMPLE 2 is digested with NruI and BstEII
resulting in a BPTI encoding DNA fragment with one blunt end
(NruI). The fragment was then monodirectionally cloned into
the SspI BstEII pMAD5-ATF-BPTI vector. The thus constructed
25   plasmid named pMAD5-ATF-BPTI-BPTI was used as a shuttle
vector for the construction of recombinant adenoviruses.
            This approach can be repeated multiple times to
construct vectors containing multiple BPTI-domains.

30   EXAMPLE 8
            A vector encoding a hybrid protein containing both
a BPTI unit and a TIMP1 unit coupled to the ATF domain has
been constructed. To construct this BPTI-TIMP vector, the
following strategy is followed.
35   The pMAD5-ATF-BPTI described in EXAMPLE 4 is
digested with the restriction enzymes SspI and BstEII. In

this way the vector is opened right behind the BPTI sequence. The pCRII-TIMP plasmid described in EXAMPLE 6 is digested with NruI and BstEII resulting in a TIMP1 encoding DNA fragment with one blunt end. The fragment was then cloned into

5   the SspI BstEII pMAD5-ATF-BPTI vector. The thus constructed plasmid named pMAD5-ATF-BPTI-TIMP was used as a shuttle vector for the construction of recombinant adenoviruses.

EXAMPLE 9

10   To monitor the production of a functional ATF-BPTI hybrid protein after transfection of cells with pMAD5 or transduction with a recombinant replication-deficient ATF-BPTI encoding adenovirus, the following tests have been performed.

15   -   The production of the hybrid ATF-BPTI protein by CHO cells transfected with the pMAD5-ATF-BPTI was analyzed using a uPA ELISA that recognizes the ATF, the aminoterminal fragment of u-PA. Production of ATF-BPTI was clearly detectable both after transient transfection of CHO cells

20   with the pMAD5-ATF-BPTI plasmid (50-100 ng/ml/24hrs) and after transduction with an ATF-BPTI encoding adenoviral vector (up to 1.5 µg/ml/24hrs).

     -       The cell culture media of CHO cells transduced with the ATF-BPTI adenovirus were analyzed using western blotting

25   techniques. After electrophoresis and blotting, parallel filters were analyzed with polyclonal antibodies against either u-PA or BPTI (raised against Trasylol®). In both filters a signal was detected at the same expected position at approximately 20kDa. This indicates that the protein

30   produced indeed contains fragments of u-PA and BPTI, thus that the hybrid protein is produced.

     -       The function as an inhibitor of plasmin activity of the ATF-BPTI protein was first analyzed in solution using dilutions of the culture medium of ATF-BPTI virus infected

35   CHO cells (approximately 1.8 µg/ml). They were incubated with plasmin (1 nM) and the activity of plasmin was measured using

a chromogenic substrate. Trasylol® dilutions were used as
control references. Plasmin inhibition by ATF-BPTI medium was
very effective, diluting the medium 1000x (i.e. 100 nM ATF-
BPTI) resulted in a 50% inhibition of the activity of 1 nM
5   plasmin, a similar inhibition as was observed with 100 nM
Trasylol®. Thus the activity of ATF-BPTI is comparable to
that of commercially available Trasylol® (Bayer, Germany).
-            The function of ATF-BPTI as an inhibitor for
plasmin bound to the cell surface via the interaction of the
10   ATF domain with the u-PA receptor (uPAR) was tested using
mouse cell lines that are either or not transfected with the
human uPA receptor gene. These cells were incubated for 6 hrs
with diluted medium of the ATF-BPTI virus-infected CHO cells.
Cell extracts were made of the uPAR-transfected cells and the
15   parental mouse cells lacking the human uPAR. Parallel
cultures underwent a short acid treatment (pH 3, 3 min)
before the cell extracts were made. This treatment will
remove any u-PA or ATF bound to the u-PA receptor. The cell
extracts were incubated with 1nM plasmin and the plasmin
20   activity was determined. Plasmin activity could only be
inhibited by the cell extract of the u-PAR containing cell
line. No inhibition of plasmin activity was observed in the
cell extracts of parental cell line, lacking the u-PA
receptor, and in the acid-treated u-PAR containing cell line.
25   This clearly indicates that ATF-BPTI can function as a u-PAR
bound plasmin inhibitor.

TABLE 1

	% INHIBITION OF PLASMIN ACTIVITY			
cell line	uPAR transfected cell line		parental cell line	
acid treatment	−	+	−	+
% inhibition	93%	0%	0%	0%

EXAMPLE 10

     Cell-specific expression of ATF-BPTI in endothelial cells e.g. to specifically inhibit the migration of endothelial cells during angiogenesis, is achieved by cloning
5   sequences of the promoter of the human pre-pro-endothelin 1 gene (nucleotide 2180-3680 of HUMEDN1B (GENBANK)) in front of the ATF-BPTI encoding DNA in an adenoviral vector. In this way, highly endothelial cell-specific expression of the ATF-BPTI hybrid protein can be obtained.
10

EXAMPLE 11

     Proteolytic degradation of the extracellular matrix is a key event in many cell migration and tissue remodeling processes. This proteolytic matrix degradation is often found
15   to be mediated by urokinase-type plasminogen activation. In order to test whether infection with an ATF-BPTI encoding adenovirus can inhibit plasmin mediated extracellular matrix degradation, an experiment was performed using human synoviocytes. These cells were infected with an ATF-BPTI
20   adenovirus while they were seeded on an ^{3}H-labeled extracellular matrix existing of bovine cartilage material. Profound inhibition of matrix degradation could be observed in the virus treated cells (figure 4) indicating that matrix degradation can be inhibited by infecting cells with the ATF-
25   BPTI encoding virus.

     Figure 4 shows the degradation of cartilage matrix by human synoviocytes in the presence of plasminogen. Matrix is incubated with control medium (lane 1), synoviocytes (lane 2), synoviocytes infected with ATF-BPTI adenovirus (lane 3),
30   and synoviocytes incubated with Trasylol®(100KIU/ml)(lane 4).

EXAMPLE 12

     In the process of restenosis smooth muscle cell migration and vessel wall remodeling are key events in which
35   plasmin mediated proteolysis of extracellular matrix components is involved. In vivo application of general

plasmin inhibitors to interfere in this process may have systemic side effects. Application of a plasmin inhibitor to the surface of the migrating cells might prevent these side effects. Infection of the blood vessel wall with an ATF-BPTI

5 adenovirus at a site where neointima formation can be expected, e.g. in a transplanted "coronary by-pass" graft, might be a ideal way to produce the ATF-BPTI locally, and thus inhibit plasmin activity in the direct surroundings of the migrating (smooth muscle) cells, resulting in a reduced

10 neointima formation.

This principle was tested using human saphenous vein organ cultures, a model system in which neointima formation can be mimicked very realistically. In parallel cultures, either or not infected with an ATF-BPTI adenovirus,

15 the neointima formation was analyzed after three and four weeks. In the untreated tissues a clear neointima formation could be observed. Profound inhibition of the neointima formation could be observed in the tissues treated with 10^{10} pfu/ml ATF-BPTI adenovirus.

Appendix

Description and Nucleotide sequence of the pMAD5-ATF-BPTI
plasmid.

5

From	To	Description
1	184	adenovirus sequence 5'
184	447	adenovirus Major Late Promoter (MLP)
447	644	tripartite leader sequence (TPL)
685	1167	urokinase ATF sequence
1168	1353	bovine prancreas trypsin inhibitor sequence
1360	1443	urokinase 3' sequence (including stop codon)
1514	1615	sequence derived form pSP65 and LacZ
1616	1751	SV40 poly A signal
1752	7334	adenovirus sequence 3'
9831	8971	β-lactamase

20 Nucleotide sequence:

```
  1    CATTTTCGCG GGAAAACTGA ATAAGAGGAA GTGAAATCTG AATAATTTTG TGTTACTCAT
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7321  CTCTTCCTCC TGGAGATCAA TTGAAGCTAG CTTTAATGCG GTAGTTTATC ACAGTTAAAT
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10321 GTAAGATTTG GC
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Claims

1.   A recombinant nucleic acid molecule comprising a vector useful for transfection or transduction of mammalian, e.g. human, cells, wherein said vector contains a nucleic acid insertion encoding an expressible hybrid polypeptide or
5 protein which comprises a domain with a binding function and a domain with an effector function.

2.   A recombinant nucleic acid molecule according to Claim 1, wherein said domain with a binding function comprises a receptor binding domain.

10 3.   A recombinant nucleic acid molecule according to Claim 2, wherein said receptor binding domain is selected from the group consisting of urokinase receptor binding domain of urokinase, receptor binding domain of epidermal growth factor, receptor associated protein that binds to LDL

15 Receptor related protein (α_2-macroglobulin receptor) and VLDL Receptor.

4.   A recombinant nucleic acid molecule according to Claim 2, wherein said receptor binding domain comprises the aminoterminal part of urokinase which is capable of binding

20 to the urokinase receptor.

5.   A recombinant nucleic acid molecule according to Claim 2, wherein said receptor binding domain comprises amino acid residues 1 through 135 of urokinase.

6.   A recombinant nucleic acid molecule according to

25 Claim 1, wherein said domain with an effector function is an enzymatically active domain.

7.   A recombinant nucleic acid molecule according to Claim 1, wherein said domain with an effector function has protease inhibitor activity.

30 8.   A recombinant nucleic acid molecule according to Claim 7, wherein said domain having protease inhibitor activity comprises a protease inhibitor or active part

thereof, said protease inhibitor being selected from the
group consisting of (bovine) pancreatic trypsin inhibitor,
(bovine) splenic trypsin inhibitor, urinary trypsin
inhibitor, tissue inhibitor of matrix metalloproteinase 1,

5   tissue inhibitor of matrix metalloproteinase 2, tissue
inhibitor of matrix metalloproteinase 3, and elastase
inhibitor.

9.   A recombinant nucleic acid molecule according to
Claim 7, wherein said domain having protease inhibitor

10   activity comprises (amino acid residues 53 through 94 of)
mature bovine pancreatic trypsin inhibitor.

10.   A recombinant nucleic acid molecule according to
Claim 7, wherein said domain having protease inhibitor
activity comprises bovine splenic trypsin inhibitor.

15   11.   A recombinant nucleic acid molecule according to
Claim 7, wherein said domain having protease inhibitor
activity comprises a tissue inhibitor of matrix metallo-
proteinases.

12.   A recombinant nucleic acid molecule according to

20   Claim 1, wherein said domain with an effector function
comprises (an active part of) two or more different protease
inhibitors, or two or more copies of (an active part of) a
protease inhibitor, or both.

13.   A recombinant nucleic acid molecule according to

25   Claim 1, wherein said vector is selected from the group
consisting of viral and non-viral vectors useful for
transfection or transduction of mammalian cells.

14.   A recombinant nucleic acid molecule according to
Claim 1, wherein said vector is an adenovirus vector or a

30   retrovirus vector useful for transfection or transduction of
human cells.

15.   A recombinant nucleic acid molecule according to
Claim 1, wherein said vector is an adenovirus vector based on
shuttle vector pMADS.

35   16.   A recombinant nucleic acid molecule according to
Claim 1, wherein said nucleic acid insertion encoding an

expressible hybrid polypeptide or protein is under the
control of a cell- or tissue-specific promoter.

17.   A recombinant nucleic acid molecule according to
Claim 1, wherein said nucleic acid insertion encoding an
5   expressible hybrid polypeptide or protein is under the
control of an endothelial cell-specific promoter, or a
vascular smooth muscle cell-specific promoter, or a liver-
specific promoter.

18.   A process for preventing local proteolytic activity,
10 extracellular matrix degradation, cell migration, cell
invasion, or tissue remodeling, comprising transfecting or
transducing the cells involved or cells in their environment
with a recombinant nucleic acid molecule as claimed in any
one of the preceding Claims to obtain local expression of the
15 hybrid polypeptide or protein encoded by said nucleic acid
molecule.

19.   A process for producing a hybrid polypeptide or
protein which comprises a domain with a binding function and
a domain with an effector function, comprising transfecting
20 or transducing mammalian cells with a recombinant nucleic
acid molecule as claimed in any one of Claims 1 to 17 to
obtain expression of the hybrid polypeptide or protein
encoded by said nucleic acid molecule, and optionally
recovering the hybrid polypeptide or protein produced.

25

Title: Method and Construct for inhibition of cell migration

Abstract

A recombinant nucleic acid molecule comprising a
vector useful for transfection or transduction of mammalian
cells, wherein said vector contains a nucleic acid insertion
encoding an expressible hybrid polypeptide or protein which
comprises a domain with a binding function and a domain with
an effector function. The domain with a binding function may
comprise a receptor binding domain, and the domain with an
effector function may have enzymatic activity, in particular
protease inhibitor activity. The vector may be a viral (e.g.
adenovirus or retrovirus) or non-viral vector useful for
transfection or transduction of mammalian cells. The nucleic
acid insertion encoding an expressible hybrid polypeptide or
protein may be under the control of a cell- or tissue-
specific promoter.

A process for preventing local proteolytic activity,
extracellular matrix degradation, cell migration, cell
invasion, or tissue remodeling, comprising transfecting or
transducing the cells involved or cells in their environment
with the recombinant nucleic acid molecule to obtain local
expression of the hybrid polypeptide or protein encoded
thereby.

A process for producing the hybrid polypeptide or
protein by transfecting or transducing mammalian cells with
the recombinant nucleic acid molecule to obtain expression
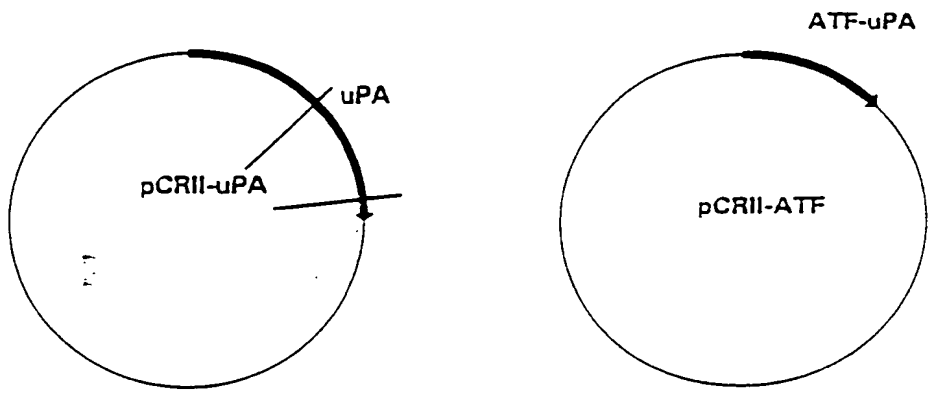and optionally recovering the hybrid polypeptide or protein
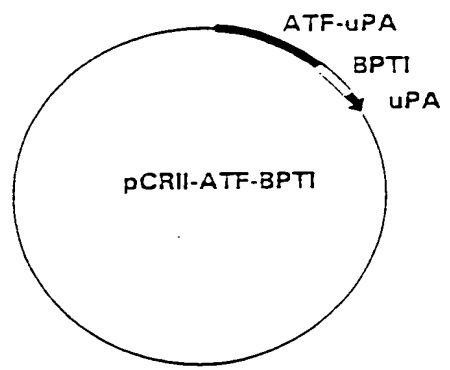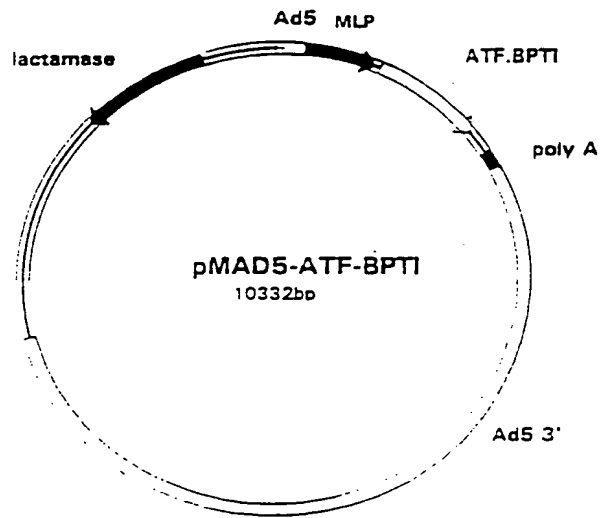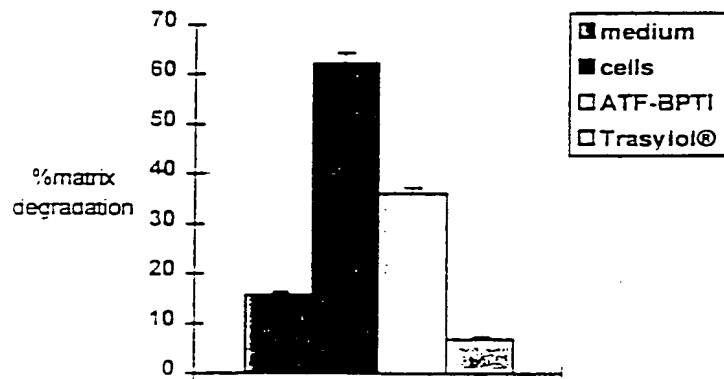produced.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4